PathNet™ Transcriptional Reporter Lentivectors

Cat. #s TR100PA-1 – TR799PA-1

PathNet™ TRE Cloning Kits

Cat. #s TR100A-1, TR500A-1

User Manual

Store kit at -20°C on receipt

A limited-use label license covers this product. By use of this product, you accept the terms and conditions outlined in the Licensing and Warranty Statement contained in this user manual.

(ver. 10-070201)

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I. Introduction and Background

A. Overview

This manual provides details and information necessary to develop PathNet™ Transcriptional Reporter (TR) constructs in lentiviral vectors (pTRH1-mCMV-dscGFP and pTRF1-mCMV-dscGFP Reporter Vectors). Specifically, it provides instructions on designing and synthesizing Transcriptional Response Element (TRE) inserts, cloning TREs into the pTRH/pTRF Vectors, and confirming successful cloning. This manual does not include information on packaging pTRH or pTRF Vector constructs into pseudoviral particles or transducing your target cells of your choice with these particles. This information is available in the user manual provided with the Lentivector Packaging Kit from SBI (Multiple Cat. #s) which is available on the SBI web site (www.systembio.com). Before using the reagents and materials supplied with this system, please read the entire manual.

B. Lentiviral Transcriptional Reporter System

Advantages of lentivector technology include:

- Ready-to-use pre-packaged constructs with a wide range of Transcriptional Response Elements (TREs) for multiple transcriptional factors.
- Lentiviral reporter constructs can efficiently transduce nearly all cell types, even those that are difficult-to-transfect such as primary or non-dividing mammalian cells.
- Our lentiviral-based reporter system is a novel approach to study transcriptional regulation and offers many advantages over current transcription reporter systems. TR constructs will integrate into the genome and therefore be subject to chromatin regulation (Leung, T.H., *et.al.,* 2004). Expression of the reporter gene indicates activation of a given transcriptional response element (TRE) by the cognate transcriptional factor in the natural chromosomal environment rather than in the episomal state in the nucleoplasm as is the case for conventional plasmid-based TR vectors. Tandem copies of integration can be avoided, thus allowing for faithful promoter regulation. Copy number of reporter constructs can be controlled by varying the multiplicity of infection (MOI).
- Construction of stable reporter cell lines is possible with TR lentivectors in just several days without the need for conventional, low efficiency selection of stable transfectants.
- Monitoring of signaling pathways by flow cytometry (FACS) is enabled by GFP reporters.

SBI's basic PathNet™ TR lentivectors contain three different reporters (dscopGFP, Luciferase and β-Galactosidase) under the control of the minimum (m)CMV promoter. mCMV is not active without a TRE specific to a transcriptional factor (Figure 1). Vectors with mCMV can be used as negative controls in the experiment or as vectors for cloning any TRE of interest. After the introduction of a specific TRE upstream of mCMV, this construct becomes a fully active promoter and expression of the reporter gene depends on the activity of the specific TF (Figure 1).

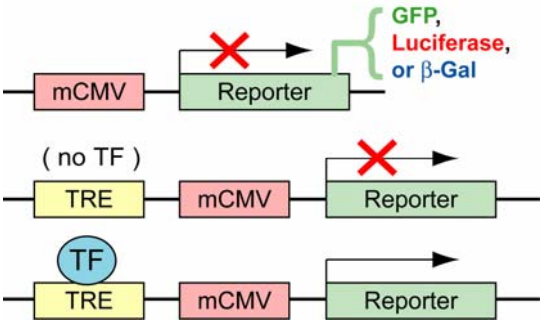

Fig. 1. Activity of the vectors depends on promoter sequence and presence of a specific transcriptional factor.

TR vectors usually contain several repeats of specific transcriptional response elements (for Cat.#s see table below) to ensure efficient activation of the minimum promoter due to cooperative interaction between TF molecules.

The pTR-dscGFP vectors contain a destabilized (ds) copGFP reporter gene under the CMV promoter and WPRE element. dscGFP is a novel fluorescent protein, derived from copepod plankton (Panalina sp.), which is similar to EGFP but has a brighter color (see Appendix). A unique feature of this protein is the presence of an additional destabilizing peptide on the C-end of the protein which shortens the half life time of the mature protein without additional transcription to 1 hour. The two other reporters (Luciferase and β-Galactosidase) have codon usage optimized for translation in Human cells.

C. Transcriptional Response Elements

Unfortunately, TRE sequences for any transcriptional factor in different promoters are not conserved. TRE sequences can be found in the TRANSFAC professional Data Base (www.biobase.com) or in the public version of this Data Base (www.gene-regulation.com). Because each transcriptional factor has multiple TREs derived from different promoters we recommend to use a consensus sequence for each particular factor to make a reporter construct. However, this consensus sequence may not be the most efficient recognition element for all cell types.

SBI transcriptional reporter vectors are designed for simple, rapid, and convenient assessment of the *in vivo* activation of signal transduction pathways. SBI has created a series of inducible reporter plasmids that contain three different reporter genes driven by a minimum CMV promoter plus a defined inducible response element (see Appendix). These plasmids are particularly suited for the *in vivo* readouts of signal transduction pathways since these TFs are convergent points of many signal transduction pathways. Because the lentiviral sequences are integrated into the genome, activation of reporter gene expression reflects changes in activity of the desired transcriptional factors in a natural chromatin environment, in comparison to commonly used plasmid transfection, most of which stay in the cytoplasm. These lentiviral systems are useful in studying the *in vivo* effects of a new gene, growth factor, or drug candidate on the signaling pathway. Basic reporters may also be used for cloning novel sequences in order to identify possible response elements.

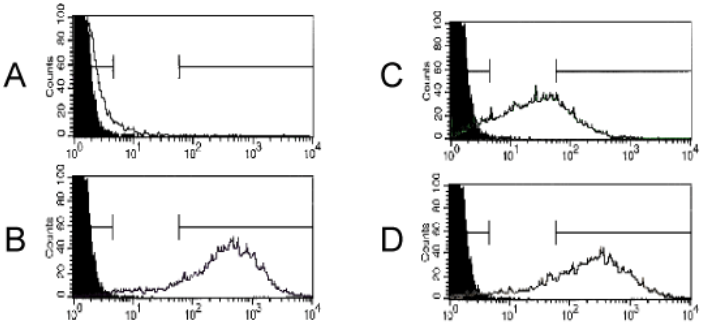

Figure 2. Analysis of p53 activity in HeLa cells transduced with different TR constructs. **A.** pTRH1-mCMV-dscGFP (negative control). **B.** pTRH1-CMV-dscGFP (positive control). **C.** pTRH1-p53-dscGFP (basal level of p53). **D.** pTRH1-p53-dscGFP (p53 was activated by PRES-229 treatment).

For your convenience, SBI provides control TR vectors and constructs for several TFs in ready-to-use form as pseudoviral particles. If you prefer to do the packaging step yourself, SBI supplies the packaging plasmids which can be purchased separately. Additional TF-specific constructs of your choice packaged in

pseudoviral particles or provided as a plasmid are available as a custom service.

D. List of Components

PathNet™ Transcriptional Reporter Vector Cloning Kits:

pTRF1-mCMV-dscGFP TRE Cloning Kit Cat. # TR100A-1
pTRH1-mCMV-dscGFP TRE Cloning Kit Cat. # TR500A-1

Component	Conc.	Amount
pTR-mCMV-dscGFP	200 ng/μl	10 μg
c-Fos TRE Ligation Control	100 nM	10 μl
Forward (cPPT) PCR Primer	10 μM	50 μl
Reverse2 (mCMV) PCR Primer	10 μM	50 μl

FIV-Based PathNet™ Transcriptional Reporter Vectors (plasmid):

dscGFP Reporter Vector	Catalog #
pTRF1-mCMV-dscGFP	TR100PA-1
pTRF2-CMV-dscGFP	TR101PB-1
pTRF1-p53-dscGFP	TR102PA-1
pTRF1-NFkB-dscGFP	TR103PA-1
pTRF1-AP1-dscGFP	TR104PA-1
pTRF1-cFos-dscGFP	TR105PA-1
pTRF1-cJun-dscGFP	TR106PA-1
pTRF1-FosB-dscGFP	TR107PA-1
pTRF1-JunD-dscGFP	TR108PA-1
pTRF1-DP1-dscGFP	TR109PA-1
pTRF1-ErbA-dscGFP	TR110PA-1
pTRF1-REVERB-dscGFP	TR111PA-1
pTRF1-Sp1-dscGFP	TR112PA-1
pTRF1-Sp3-dscGFP	TR113PA-1
pTRF1-NMyc-dscGFP	TR114PA-1

Luciferase Reporter Vector	Catalog #
pTRF3-CMV-Luc	TR201PC-1
pTRF2-p53-Luc	TR202PB-1
pTRF2-NFkB-Luc	TR203PB-1
pTRF2-AP1-Luc	TR204PB-1
pTRF2-cFos-Luc	TR205PB-1
pTRF2-cJun-Luc	TR206PB-1
pTRF2-FosB-Luc	TR207PB-1
pTRF2-JunD-Luc	TR208PB-1
pTRF2-DP1-Luc	TR209PB-1
pTRF2-ErbA-Luc	TR210PB-1
pTRF2-REVERB-Luc	TR211PB-1
pTRF2-Sp1-Luc	TR212PB-1
pTRF2-Sp3-Luc	TR213PB-1
pTRF2-NMyc-Luc	TR214PB-1

All plasmids are shipped at a concentration of 200 ng/μl and an amount of 25 μg. All kits are shipped in dry ice and should be stored at -20°C upon receipt. Properly stored kits are stable for 12 months from the date received.

For the complete list of available transcriptional reporter vectors, please visit our website at www.systembio.com

HIV-Based PathNet™ Transcriptional Reporter Vectors (plasmid):

dscGFP Reporter Vector	Catalog #
pTRH1-mCMV-dscGFP	TR500PA-1
pTRH1-CMV-dscGFP	TR501PA-1
pTRH1-p53-dscGFP	TR502PA-1
pTRH1-NFkB-dscGFP	TR503PA-1
pTRH1-AP1-dscGFP	TR504PA-1
pTRH1-cFos-dscGFP	TR505PA-1
pTRH1-cJun-dscGFP	TR506PA-1
pTRH1-FosB-dscGFP	TR507PA-1
pTRH1-JunD-dscGFP	TR508PA-1
pTRH1-DP1-dscGFP	TR509PA-1
pTRH1-ErbA-dscGFP	TR510PA-1
pTRH1-REVERB-dscGFP	TR511PA-1
pTRH1-Sp1-dscGFP	TR512PA-1
pTRH1-Sp3-dscGFP	TR513PA-1
pTRH1-NMyc-dscGFP	TR514PA-1

Luciferase Reporter Vector	Catalog #
pTRH2-CMV-Luc	TR601PB-1
pTRH2-p53-Luc	TR602PB-1
pTRH2-NFkB-Luc	TR603PB-1
pTRH2-AP1-Luc	TR604PB-1
pTRH2-cFos-Luc	TR605PB-1
pTRH2-cJun-Luc	TR606PB-1
pTRH2-FosB-Luc	TR607PB-1
pTRH2-JunD-Luc	TR608PB-1
pTRH2-DP1-Luc	TR609PB-1
pTRH2-ErbA-Luc	TR610PB-1
pTRH2-REVERB-Luc	TR611PB-1
pTRH2-Sp1-Luc	TR612PB-1
pTRH2-Sp3-Luc	TR613PB-1
pTRH2-NMyc-Luc	TR614PB-1

All plasmids are shipped at a concentration of 200 ng/μl and an amount of 25 μg. All kits are shipped in dry ice and should be stored at -20°C upon receipt. Properly stored kits are stable for 12 months from the date received.

For the complete list of available transcriptional reporter vectors, please visit our website at www.systembio.com

Transcriptional Response Elements (TREs)

Transcriptional factor	Recognition element
AP-1	(TCAGTCAG)6
C/EBPalpha	(TTACGTCA)6
c-Fos	(GGTGTAA)6
c-Jun	(GTGACGTCAC)6
c-Myc	(CGTGGTCGACCACGTGGTCGACCACGTGGTCGACCACGTGACCA)2
c-Rel	(GGGGAATCTCCCGGGGAATCTCCC)3
DP-1	(ATTGGCGCGAAAtAAAAATTGGCGCGAAA)2
E2F+p107	(TCGCGG)6
E2F-1	(TTTCCCGC)6
E2F-4/DP-2	(GGTTTTCCCGCCTTTT)4
Egr-1	(CACCCCCAC)6
ErbA	(TCAGGTCA)6
FosB	(TGTAATA)4
HIF-1	(TACGTG)4
HSF1	(TCTAGAAG)6
INF	(TTTCtcTTTCAG)5
JunD	(GGTGTAATA)6
Max1	(ACGTGGTCGACCACGTGGTCGACC)3
NF-kB	(GGGACTTTCC)4
N-Myc	(AACATCAGCCCCCCACGTGATACAACATCAGC)2
p53	(ACATGTCCCAACATGTTGTCG)8
REVERB-alpha	(AGGTCA)6
Sp1	(6GGGGCGGCGC)6
Sp3	(GGCCCTGCCCTC)3
SRF	(CCATATATGG)3
YY1	(CCAAATATGG)4

E. Additional Required Materials

For vectors purchased in plasmid form, you will also need to purchase the appropriate pPACK Lentivector Packaging Kit (Cat. # LV100A-1 for FIV-based vectors and Cat. # LV500A-1 for HIV-based vectors) and the 293TN Producer Cell Line (Cat. # LV900A-1) or HEK 293T cells (ATCC, Cat. # CRL-11268) in order to package your reporter constructs into pseudoviral particles. The protocol for packaging and transduction of packaged pseudoviral particles is provided in the SBI Lentivector Expression Systems User Manual and manual for HEK 293T cells (ATCC, Cat. # CRL-11268).

Packaging Plasmid Kit	Origin	Catalog #
pPACKF1	FIV-based	LV100A-1
pPACKH1	HIV-based	LV500A-1

For Annealing TRE Oligonucleotides

- 2X DNA Annealing Buffer
  (20mM Tris, pH 7.8; 100mM NaCl; 0.2mM EDTA)

For Phosphorylation of Annealed Oligonucleotides

- T4 Polynucleotide Kinase
  (Recommended: New England BioLabs T4 Polynucleotide Kinase, Cat. # M0201S)

For Ligating and Transforming Reporter Vector Construct

- T4 DNA Ligase and ligation reaction buffer
  (Recommended: New England BioLabs T4 DNA Ligase, Cat. # M0202S. Dilute to 5 U/μl with the provided 1X reaction buffer just before use)
- Competent *E. coli* cells (RecA$^-$)
  (Recommended: Invitrogen One Shot OmniMAX 2 T1 Phage-Resistant competent cells, Cat. # C8540-03)
- Petri plates containing LB Agar media with 50 μg/ml Ampicillin

For Screening Inserts and sequencing.

- Taq DNA polymerase, reaction buffer, and dNTP mix
  (Recommended: Clontech Titanium™ Taq DNA polymerase, Cat. # 639208)
- PCR machine
- Forward and reverse primers (see Appendix for the sequences).
- 2-3% 1X TAE Agarose gel

For Purifying Reporter Vectors after Cloning

- Plasmid purification kit
  (Recommended: QIAGEN Endotoxin-free Plasmid Maxi Kit, Cat. # 12362. The following combination of kits can be used for Midi scale preparation of endotoxin-free DNA:
  - QIAfilter Plasmid Midi Kit, Cat. # 12243, and EndoFree Plasmid Buffer Set, Cat. # 19048

  Please visit the QIAGEN website to download the specialized protocol that is not contained in the current user manual:
  - http://www1.qiagen.com/literature/protocols/pdf/QP15.pdf

F. Safety Guidelines

Work with FIV-based and HIV-based lentiviral vectors falls within NIH Biosafety Level 2 criteria. For a detailed description of laboratory biosafety level criteria, consult the following pages on the Centers for Disease Control Office of Health and Safety Web site:

http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s3.htm
http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

Also, you should consult the health and safety guidelines and officers at your institution regarding use and handling of the lentiviral system. In addition, although the system itself has been designed to minimize

possible risk, specific recombinant lentivector constructs may be potentially hazardous, depending on the nature of introduced insert (such as oncogenes, toxins, Transcriptional Response Element to tumor suppressor genes, etc.). For these reasons, it is critical to exercise due caution while working with recombinant lentiviruses.

To ensure safe laboratory handling, you should thoroughly understand the biology of the lentiviral vectors and the specific modifications and design features of the SBI system with which you are working. The original FIV viral vector was developed by Eric M. Poeschla, David J. Looney, and Flossie Wong-Staal in UCSD (Poeschla 2003). Based on this original pFIV vector, the FIV-based pTRF Vectors for cloning and delivering TREs into cells were developed at SBI. These lentivectors have been modified to remove sequences that overlap with the packaging plasmid to minimize the possibility of homologous recombination and generation of self-replicating viral sequences when co-transfecting these constructs into packaging cells. SBI's lentivectors also have a deletion in the enhancer of the U3 region of 3' ΔLTR to ensure self-inactivation of the lentiviral vector after transduction and integration of the sequences into the genomic DNA of the target cells.

SBI's pTRH lentivectors together with the pPACKH1 packaging plasmids comprises a third-generation HIV-1-based cloning vector system. These lentivectors are based on the vectors developed for gene therapy applications by Dr. J. G. Sodroski (US patent #5,665,577 and # 5,981,276). This system is designed to maximize its biosafety features including:

- Deletion in the enhancer of U3 region of 3'LTR ensures self-inactivation of lentiviral construct after transduction and integration into genomic DNA of the target cells.
- RSV promoter upstream of 5'LTR in pTRH expression vector allows efficient Tat-independent production of viral RNA, reducing the number of genes from HIV-1 that are used in this system.
- Number of HIV-1 viral genes necessary for packaging, replication and transduction is reduced to three (gag, rev and pol), and these genes are expressed from different plasmids lacking packaging signals and significant homology to pSIH expression vectors, VSV-G expression vector, or each other to prevent generation of recombinant replication-competent virus.
- None of the HIV-1 genes (*gag, pol, rev)* will be present in the packaged viral genome, as they are expressed from packaging plasmids lacking packaging signal—therefore, the lentiviral particles generated are replication-incompetent.
- Pseudoviral particles will carry only the expression construct of your target gene.

To avoid any possible contamination and maintain a clean laboratory environment we also recommend following these standard safety practices:

- Wear double gloves, face protection, and lab coat at all times.
- Perform work in a limited access area in a Biological Safety Cabinet Class II and post biohazard warning signs.
- Minimize splashes or aerosols with careful pipetting.
- Take precautions with needles, blades, etc.
- Decontaminate work surfaces at least once a day and after any spill of viable material.
- Decontaminate all cultures, stocks, and other biological wastes before disposal using approved decontamination methods, such as autoclaving. Before decontamination the biological materials should be placed in a sealed, durable, leak-proof container for transport from the laboratory.

II. Protocol

A. Transcriptional Response Element Oligonucleotide Design and Synthesis

Typically, 4 or 5 repeats of TRE sequences of interest should be inserted upstream of the mCMV promoter for efficient activation of transcription. Although there is no standard rule for selecting TRE sequences for particular factor, we recommend using consensus sequences (for example, see **http://bio.chip.org/mapper** or **http://mars.kribb.re.kr:8080/tfExplorer/** websites) or a TRE from a promoter for genes which are highly expressed in most cell types or tissues. You should also consider that the size of each recognition element may be different, but it is preferable to have them one or two helix turns apart, *i.e.* 11 or 22 nucleotides, in order to be located on the same side of the DNA helix. If necessary, add an extra spacer between TRE repeats to follow this rule.

For each selected sequence, two complementary TRE oligonucleotides (a top and a bottom strand) need to be synthesized, then annealed before the phosphorylation and ligation steps. Below are guidelines for synthesis of the Transcriptional Response Element oligonucleotides:

(1) A 50 nmol scale reaction for DNA oligonucleotide synthesis with regular desalting purification is sufficient for cloning into the TR lentivectors.

(2) For the best cloning efficiency, we recommend using phosphorylated oligonucleotides which can typically be ordered from the supplier. Alternatively, you can phosphorylate the oligonucleotides after synthesis using T4 polynucleotide kinase. The phosphorylation protocol is provided below in step B.2.

(3) In addition to the Transcriptional Response Element sequence, the oligonucleotide needs to include a 2- or 4-base sequence at the 5' end of each oligonucleotide which will be complementary to the desired cloning sites in the pTR vector. Typically, we clone into the EcoR1 and Spe1 sites (see diagram below). These sequences form "sticky-ends" that facilitate ligation with the linear vector. The annealed sequences should have a double-stranded Transcriptional Response Element structure as shown in the following diagram.

Top strand

```
5′- AATTNNNNNNNNNNNNNNNNNNN      -3′
3′-      NNNNNNNNNNNNNNNNNNNGATC -5′
```

Bottom strand

B. Cloning of TRE into pTR Vector

1. Anneal TRE Oligonucleotides

a. Dissolve the TRE oligonucleotides in an appropriate amount of deionized water to a final concentration of 1 μM.

b. Prepare the ds TRE oligonucleotide as follows:

2.5 μl	Top strand TRE oligonucleotide
2.5 μl	Bottom strand TRE oligonucleotide
25.0 μl	2X Annealing Buffer
20.0 μl	Deionized water
50.0 μl	Total volume

c. Heat the mixture to 95°C for 5 min in a thermocycler or heating block.

d. Turn off the thermocycler or heating block and let it cool to room temperature.

e. The annealed oligonucleotide (100 nM) is ready for the phosphorylation and ligation steps. Store the annealed oligonucleotides at -20°C until use.

2. Phosphorylate the Transcriptional Response Element Duplex

Note: If your oligonucleotides are already phosphorylated, dilute them to 10 nM in 1X Annealing Buffer, skip this phosphorylation step, and proceed to ligation in step 3. For the insert-minus control, you may either follow step 2 or use 1 μl Annealing Buffer in step 3.

a. Set up 10 μl phosphorylation reactions for each experimental TRE template as follows:

1 μl	Annealed ds TRE template oligos from step B.1 (100 nM) *
1 μl	10X T4 Polynucleotide Kinase Buffer
1 μl	10 mM ATP
6 μl	Deionized water
1 μl	T4 Polynucleotide Kinase (10 U/μl)
10 μl	Total volume

* For the insert-minus control, use 1 μl 1X Annealing Buffer

b. Incubate the phosphorylation reaction at 37°C for 30 minutes.

c. Use 1 μl (10 nM) for the ligation step.

3. Ligate the TRE into pTR vector

a. Set up 10 μl ligation reactions for each phosphorylated TRE template as follows:

2.5 μl	Linearized Reporter Vector (20 ng/μl)
1.0 μl	Phosphorylated ds TRE template (step B2; 10 nM) **
1.0 μl	10X T4 DNA Ligase Buffer
4.5 μl	Deionized water
1.0 μl	T4 DNA ligase (5 U/μl)
10.0 μl	Total volume

** For controls, use insert-minus (from step B2) and positive ligation control TRE for c-Fos provided in the kit (Use 1 μl of positive ligation control TRE per reaction).

b. Incubate the ligation reaction at 16°C for 2-4 hrs.

4. Transform *E. coli* with the ligation product

a. For each experimental TRE template, use the whole volume of ligation reaction for transformation.

b. Follow the manufacturer's protocol for transforming the competent cells with each experimental and control TRE construct.

c. Plate an appropriate amount of transfected cells on LB plates with 50 μg/ml ampicillin and grow overnight at 37°C.

C. Identify clones with Transcriptional Response Element inserts

1. Prepare colony cultures

a. Randomly pick up 10 well-separated colonies from each plate and grow each clone in 100 μl of LB Broth with 100 μg/ml ampicillin at 37°C for 2 hours with shaking.

b. Take 1 μl of each bacteria culture for PCR screening (see C.2) and continue to grow the cells for another 6 hours.

c. Store the bacterial culture at 4°C.

2. Screen for TRE template inserts in lentiviral construct

Clones with TRE insert can be identify using the Forward cPPT PCR Primer and Reverse2 (mCMV) PCR Primer provided in the kit (see Appendix for the sequence of the primers).

a. Prepare a PCR master as follows:

1 rxn	10 rxn	Composition
0.5 µl	5 µl	Forward (cPPT) PCR primer (10 µM)
0.5 µl	5 µl	Reverse2 (mCMV) PCR primer (10 µM)
0.5 µl	5 µl	50X dNTP mix (10 mM of each)
2.5 µl	25 µl	10X PCR Reaction Buffer
19.5 µl	195 µl	Deionized water
0.5 µl	5 µl	Taq DNA polymerase (approx. 5 U/µl)
24.0 µl	240 µl	Total volume

b. Mix the master mix very well and aliquot 24 µl into each well of 96-well PCR plate or individual tubes.

c. Add 1 µl of each bacterial culture from C.1 into each well or tube.

d. Proceed with PCR using the following program:

94°C, 4 min	1 cycle
94°C, 0.5 min, then 68°C, 30 sec.	25 cycles
68°C, 3 min	1 cycle

e. Take 5 µl of the PCR reaction and run it on a 2-3% agarose/EtBr gel in 1X TAE buffer.

The expected size of the PCR product if there is no insert present is 277 bp for FIV-based copGFP vectors, 300 bp for FIV-based Luciferase vectors, 150 bp for HIV-based copGFP vectors, and 175 bp for HIV-based Luciferase vectors. With the positive control c-Fos insert, the size is an additional 74 bp. For positive clones with your insert, add the size of your insert to the appropriate size above.

Grow a positive clone with TRE insert in an appropriate amount of LB-Amp Broth, and purify the TR construct using an endotoxin-free plasmid purification kit (see Section I.F).

Confirm identity of TRE insert by sequence analysis of the pTR construct using the Forward cPPT PCR primer (for sequences, see Appendix, Section V.F).

D. Testing of the TR constructs

If you are planning to use SBI's pTR Lentiviral Vectors for viral delivery, first screen the TRE constructs generated in section C to determine their effectiveness in the cells of interest. TR constructs

can be tested as reporters in a cell based assay directly after transfection into an appropriate cell line in the same way as regular TR plasmids. To rapidly screen the lentiviral TRE constructs in plasmid form, you can deliver and express them in HeLa or HEK 293 cells using chemical transfection. For example, with these cells, the Lipofectamine™ Reagent (Invitrogen, Cat. # 18324-111) with Plus™ Reagent (Invitrogen, Cat. # 11514-015) system works well. Alternatively, you can use your target cells for this analysis. If you have already established a transfection method for your target cells, use your established conditions. If you do not have an established transfection protocol, we recommend you compare efficiencies of several transfection procedures (*e.g.*, Invitrogen's Lipofectamine™ 2000, Cat. # 11668-027, Clontech's CLONfectin™, Cat. # 631301).

For TRE activation studies using transfection, it is important to optimize the selected transfection protocol and then keep the parameters constant to ensure reproducible results. Once you identify a functional TRE construct, you can package this construct into lenti pseudoviral particles, and efficiently transduce it into any target cells of choice. For this purpose, you will need to purchase the pPACKF1 (for FIV-based pTRF constructs) or pPACKH1 (for HIV-based pTRH constructs) Lentivector Packaging Kit from SBI (see Appendix). Figure 3 schematically shows all steps which need to be performed in order to generate pseudoviral packaged Transcriptional reporter constructs. A detailed protocol with descriptions of each step can be found in the Lentivector Packaging Kit manual also available on SBI's website (www.systembio.com).

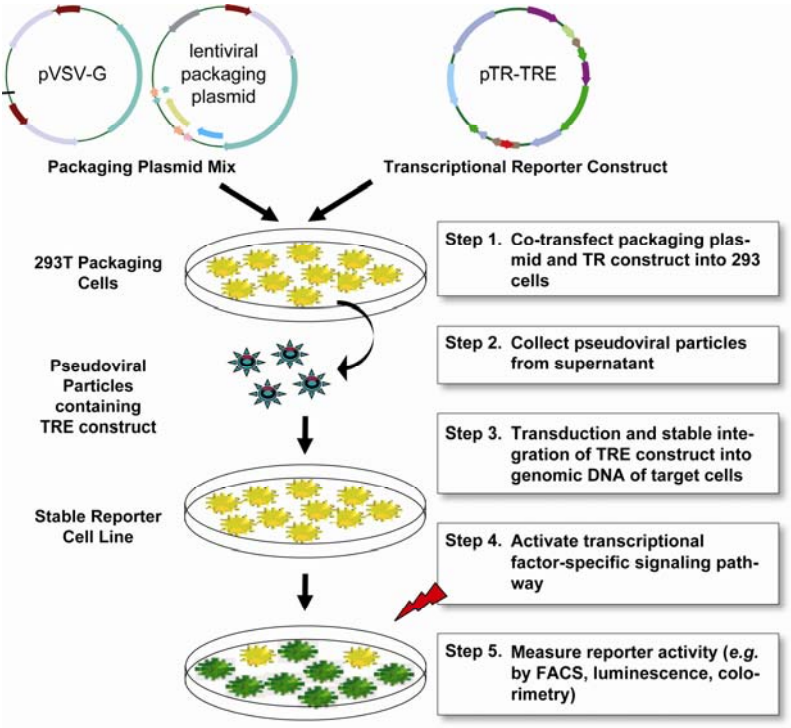

Fig. 3. Schematic presentation of the packaging procedure for pTR constructs and making of stable cell lines.

The Lentivector Packaging Kit User Manual includes the procedural information for packaging the viral vector. This user manual is also available on the SBI web site (www.systembio.com). Although you can create stable transfectants with the lentiviral construct using standard transfection and selection protocols, transduction of the lentiviral TRE construct using packaged pseudoviral particles is the most efficient way to deliver TRE constructs in a wide range of cells, including dividing, non-dividing, and hard-to-transfect cells.

III. Troubleshooting

1. Getting Few or No Clones

Use positive ligation control
If you did not use the positive control, repeat all steps (*i.e.* annealing, phosphorylation, and ligation) to check the quality of your reagents.

Check design of the TRE insert
Check the sequence of the Transcriptional Response Element oligonucleotides to ensure that, after top-bottom strand annealing, the ends present on both 5' ends overhang for proper annealing with the restricted ends of the linearized pTR Vector. Also, confirm that the top and bottom strand sequences reverse complement each other.

Check top-bottom strand annealing
To ensure a high percentage (80%) of double-stranded DNA after annealing, check the concentration of TRE oligonucleotides using a spectrophotometer and mix equal molar amounts of each strand. For optimal annealing, turn off the thermocycler after denaturation and let the tubes cool down to room temperature. Evaluate 5 μl of annealed insert (from step II.B.1.e) using a 12% polyacrylamide gel and compare the band's location with that of the original single-stranded oligonucleotides.

Confirm oligonucleotides were correctly synthesized
Verify the size of the oligonucleotides using a 12% native polyacrylamide gel.

Check quality of T4 polynucleotide kinase and T4 DNA ligase
Test the activity of your ligase and reaction buffer using a different vector and insert. Test the activity of T4 polynucleotide kinase by labeling annealed oligonucleotides with ^{32}P-γATP. Replace the reagents if they show poor activity.

Ensure there are no ligation inhibitors present
EDTA and high salt can inhibit ligation reactions. Make sure that your double strand oligonucleotide concentration is only 100 nM and that you dilute it at least 10-fold before adding it to the ligation reaction.

Check the quality of the competent cells
Handle the competent cells gently. Many cells can not be refrozen once thawed. The quality of the competent cell can be tested by transforming with any circular plasmid.

Check antibiotic selection
The plates used for cloning should contain 50-100 μg/ml ampicillin in the media.

2. No product was amplified from selected clones

Confirm activity of the Taq DNA polymerase
Test the activity of the enzyme reaction by amplifying a known sequence from any plasmid DNA. Replace the reagents if they demonstrate poor activity.

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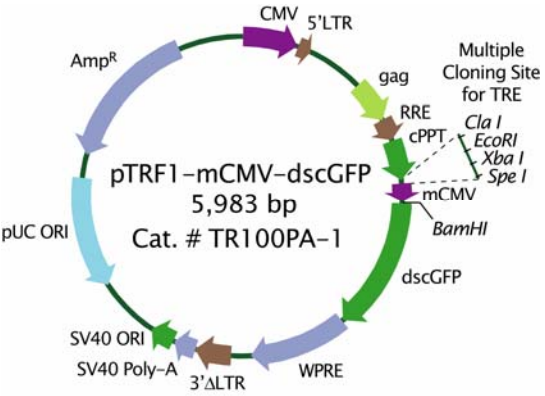
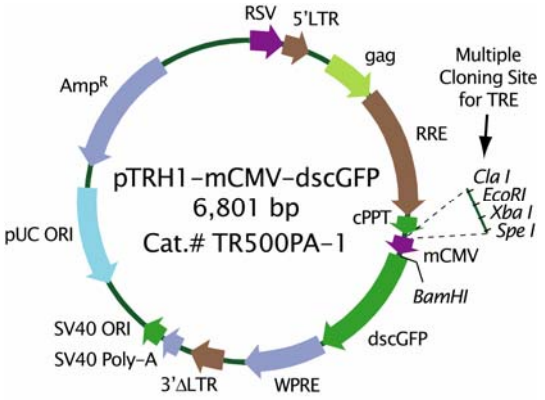
V. Appendix

A. Map of pTRF1-mCMV-dscGFP Vector



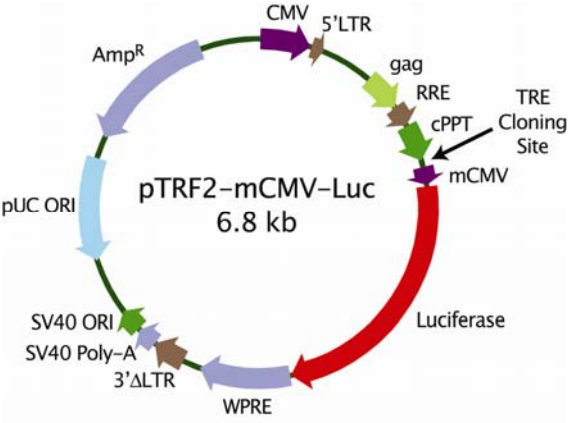
B. Map of pTRH1-mCMV-dscGFP Vector



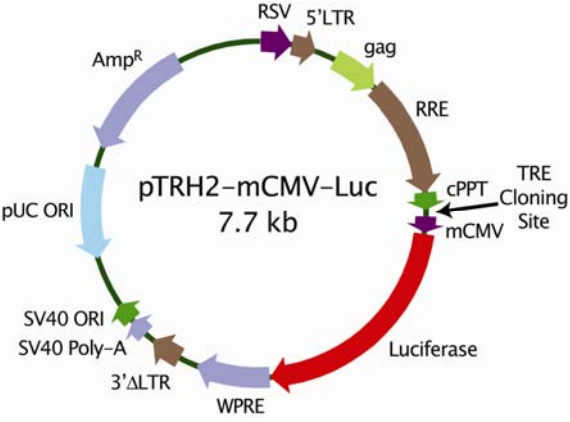
TRE Multiple Cloning Site

```
          EcoRI
         ------
   ClaI      XbaI     SpeI
  ------    ------  ------
ATTTTATCGATGAATTCTAGAACTAGTTAGG
TAAAATAGCTACTTAAGATCTTGATCAATCC
```

C. Map of pTRF2-mCMV-Luc Vector



D. Map of pTRH2-mCMV-Luc Vector



E. pTRF1-mCMV-dscGFP Features

Feature	Location*	Function
CMV-5' LTR	1-415	Hybrid CMV promoter-R/U5 long terminal repeat; required for viral packaging and transcription
gag	762-1011	Packaging signal
RRE	1012-1143	Rev response element binds gag and involved in packaging of viral transcripts
cPPT	1150-1390	Central polypurine tract (includes DNA Flap region) involved in nuclear translocation and integration of transduced viral genome
CMV promoter	1391-1740	Human cytomegalovirus (CMV)--constitutive promoter for transcription of dscGFP
dscGFP	1766-2566	Copepod green fluorescent protein (similar to regular EGFP, but with brighter color) as a reporter for the transfected/ transduced cells; a destabilizing (ds) peptide on the C-end shortens the half life time of the mature protein to 1 hour
WPRE	2575-3164	Posttranscriptional regulatory element which enhances the stability of the viral transcripts
3' ΔLTR (ΔU3)	3283-3498	Required for viral reverse transcription; self-inactivating 3' LTR with deletion in U3 region prevents formation of replication-competent viral particles after integration into genomic DNA
SV40 Poly-A	3499-3630	Transcription termination and polyadenylation
SV40 Ori	3639-3785	Allows for episomal replication of plasmid in eukaryotic cells
pUC Ori	4155-4828(C)	Allows for high-copy replication in *E. coli*
AmpR	4973-5833(C)	Ampicillin resistant gene for selection of the plasmid in *E. coli*

* The notation (C) refers to the complementary strand.

F. Sequences of the primers used for identification of the positive clones with TRE insert

(included only with PathNet™ TRE Cloning Kits)

For FIV-based pTRF vectors:
Forward (cPPT): 5'-AGAAGAGGTAGGATAGGAGGGATG-3'
Reverse2 (mCMV): 5'-CTGCTTATATAGACCTCCCACCGT-3'

For HIV-based pTRH vectors:
Forward (cPPT): 5'-GGGGTACAGTGCAGGGGAAAGAAT-3'
Reverse2 (mCMV): 5'-CTGCTTATATAGACCTCCCACCGT-3'

G. Properties of the dscGFP Fluorescent Protein

The pTR-dscGFP Vectors contain the full-length copGFP gene with optimized human codons for high level of expression of the fluorescent protein from the CMV promoter in mammalian cells. The copGFP marker is a novel natural green monomeric GFP-like protein from copepod (*Pontellina sp*.). The copGFP protein is a non-toxic, non-aggregating protein with fast protein maturation, high stability at a wide range of pH (pH 4-12), and does not require any additional cofactors or substrates. A unique feature of the dscGFP protein is the presence of an additional destabilizing (ds) peptide on the C-end of the protein which shortens the half life time of the mature protein without additional transcription to 1 hour. The copGFP protein has very bright fluorescence that exceeds at least 1.3 times the brightness of EGFP, the widely used *Aequorea victoria* GFP mutant. The copGFP protein emits green fluorescence with the following characteristics:

emission wavelength max – 502 nm;
excitation wavelength max – 482 nm;
quantum yield – 0.6;
extinction coefficient – 70,000 M^{-1} cm^{-1}

Due to its exceptional properties, copGFP is an excellent fluorescent marker which can be used instead of EGFP for monitoring delivery of FIV constructs into cells.

H. Related Products

- **PathNet™ Lentiviral Transcriptional Reporter Constructs (in Pseudoviral Packaged form)**
  - **Many (see Section I.D, or visit our website for a current list of available TR packaged constructs)**

  Provided in pre-packaged, ready-to-use pseudoviral particles, these HIV- and FIV-based reporter constructs allow you to transduce and analyze a wide variety of TF-specific constructs in a wide range of cells. Based on the highly efficient lentiviral system, the PathNet™ Transcriptional Reporter Vectors provide a convenient and cost-effective system to deliver and stably integrate sequences of your choice into the host genome.

- **PathNet™ Pooled TF Binding Site Library (in Plasmid form)** (Cat. # TR550PA-1)
  After packaging the plasmid library into pseudoviral particles, transduce the packaged PathNet™ library into target cells, select target cells with a specific phenotype, and identify TREs and corresponding transcription factors which induce the specific changes in expression levels of the reporter gene.

- **pPACK Lentivector Packaging Kits**
  - **FIV-Based: pPACKF1 Packaging Kit** (Cat. # LV100A-1)
  - **HIV-Based: pPACKH1 Packaging Kit** (Cat. # LV500A-1)

  Unique lentiviral vectors that produce all the necessary lentiviral proteins and the VSV-G envelope glycoprotein from vesicular stomatitis virus required to package pTR lentiviral constructs into pseudoviral particles. 293T cells (ATCC, Cat. # CRL-11268) transiently transfected with the Lentiviral Packaging Plasmid Mix and one of the pTR reporter vectors produce packaged pseudoviral particles containing a pTR-TRE-mCMV construct.

- **293TN Producer Cell Line** (Cat. # LV900A-1)
  The 293TN cell line is a highly transfectable derivative of the HEK293 cell line with constitutive expression of SV40 T-antigen and neo gene. Use with the pPACK Lentivector Packaging Kit to generate a high titer of pseudoviral particles containing your lentivector construct.

- **Lentivector Rapid Titer PCR Kit** (Cat. # LV950A-1 [for human cells], LV951A-1 [for mouse cells])
  Measure copy number (MOI) of integrated lentiviral constructs in genomic DNA of target cells after transduction with SBI's PathNet™ vectors or with constructs made in any of SBI's FIV or HIV-based lentivectors.

I. Technical Support

For more information about SBI products and to download manuals in PDF format, please visit our web site:

http://www.systembio.com

For additional information or technical assistance, please call or email us at:

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1616 North Shoreline Blvd.
Mountain View, CA 94043

Phone: (650) 968-2200
(888) 266-5066 (Toll Free)

Fax: (650) 968-2277

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General Information: info@systembio.com
Technical Support: tech@systembio.com
Ordering Information: orders@systembio.com

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CopGFP Reporter

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